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Commentary by

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on 'Comparative studies of bacterial cytochromes'
by M.D. Kamen and L.P. Vernon
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In the late '40s, largely owing to the unitary theory of photosynthesis associated with C.B. Van Niel, the conviction was fueled that primary processes in green plant and bacterial photometabolism were similar, if not identical. It seemed likely that the simultaneous stoichiometric coupled photooxidations and photoreductions which supported the Hill reaction in green plant chloroplasts had their analogues in bacterial chromatophores. However relevant data on enzyme composition of chloroplasts were fragmentary, at best, and wholly non-existent for chromatophores. So was my expertise with redox enzymes, such as the cytochrome *c* reductases and oxidase in mitochondria.

It was, therefore, most fortunate that at this time, Leo Vernon was available. He had learned how to prepare and handle chloroplasts while working for his doctorate with Sam Aronoff at Iowa State University, and acquired expertise with mitochondrial redox enzymes as a postdoctoral fellow at the University of Wisconsin. I did not think, at the time, that experience with mitochondrial cytochrome systems would help much in unraveling the photometabolism of *Rhodospirillum rubrum*, the microorganism we were using for the most part. I was firmly under the sway of the dogma that cytochromes, and especially cytochrome *c*, were only active in aerobic energy transduction, operating in coupled oxidative phosphorylation. The only cytochrome *c* characterized was the mitochondrial form. The possibility that it might be just a unique example of a large family of *c*-type cytochromes had occurred to nobody.

Nevertheless, Leo went ahead treating extracts of *R. rubrum* as though they were derived from liver mitochondria and almost immediately came up with the astounding finding that these extracts contained a heme protein, identical spectroscopically with mitochondrial cytochrome *c* and present in concentrations even greater than encountered in liver mitochondria. Perhaps on further reflection this was not too surprising, because *R. rubrum* chromatophores supported a dark aerobic metabolism involving oxygen uptake. However, Sidney Elsden in England, who had greeted Leo's findings with much skepticism, confirmed that a cytochrome *c* was present in the chromatophore, but in addition he found it did not serve as a substrate for the mitochondrial

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cytochrome *c* oxidase. Moreover, and most convincing, Leo and I found that a heme protein similar to *R. rubrum* cytochrome *c* also existed in large quantities in extracts from the strictly anaerobic photosynthetic bacterium *Chlorobium limicola*. At about the same time, Postgate in England and Ishimoto and his associates in Japan detected and characterized a cytochrome '*c*' in extracts from the strictly obligate anaerobic species of the sulfate-reducing *Desulfovibrio* which, in addition, was non-photosynthetic. It did not take much imagination to suspect that energy transduction by coupling to electron transfer with formation of ATP could be a process common to bacteria, whether photosynthetic or non-photosynthetic, aerobic or non-aerobic.

We still had the original problem of finding a Hill analogue in *R. rubrum* chromatophores, which we eventually succeeded in doing, but that is another story. The distraction, accidentally experienced, of finding something like mitochondrial cytochrome *c* in *R. rubrum* chromatophores led me away from a preoccupation with photometabolism and into a systematic research on bacterial cytochromes which was to occupy our laboratory for the next quarter century and to spread to other laboratories, so that now there is a vast body of data showing that the cytochromes *c* are a family of heme proteins varying in structure and concerned with bioenergetic processes. Most appear to be focused on coupling of electron transfer and proton translocation to ATP synthesis in both aerobic and anaerobic systems. Mitochondrial cytochrome is but one form of these heme proteins, structurally related only by covalent attachment of the prosthetic heme to the protein. A thorough-going generalization of concepts on the relation between structure and function has resulted from these researches.

The significance of this paper is that it was apparently the first to record a systematic attempt to investigate bacterial cytochromes *c*. We studied representative species of facultative photoheterophes and denitrifiers. Taken together with knowledge of cytochromes *c* isolated from the other known photosynthetic bacterial genera and from the sulfate-reducers, either in our laboratory or elsewhere, it could be supposed that cytochrome systems were operative in energy transduction of all photosynthetic systems, as well as in chemosynthetic systems other than those that were strictly fermentative.

Leo Vernon

This paper served as a prototype for the many others which were to make up the large body of data that now constitute the basis for a fascinating area of biochemical research – the comparative biochemistry of cytochromes, especially cytochromes *c*. Many new insights into the relation between structure and function, as seen in heme proteins, have been realized and more will come as new technologies and spectroscopics become available.

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COMPARATIVE STUDIES ON BACTERIAL CYTOCHROMES*

by

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INTRODUCTION

Interest in the occurrence and distribution of hematin compounds** in bacteria somewhat dormant since the first researches on this subject some 25 years ago, has revived markedly recently. It has been demonstrated that microorganisms, and in particular bacteria, provide excellent test material for the investigation of biological oxidation mechanisms, not only because they are well adapted for exploitation using such elegant methods as those developed by CHANCE and his co-workers[2,3], but also because they are found to be good source materials for the isolation and extraction of a large variety of hematin compounds and enzymes associated with such compounds[4,5,6,7,8].

In previous papers[7,8], it has been shown that a number of photosynthetic bacteria, including two strict anaerobes, contain cytochromes of the "*c*" type. Although these cytochromes possess a porphyrin moiety identical in character and in bonding to that of mammalian or yeast cytochrome *c*, their protein components differ with ensuing deviations in physico-chemical behaviour from that noted in the cytochrome *c* of mammalian tissue and yeast. As an example, (see[5,7]), the cytochrome *c* obtained from the photoheterotroph, *Rhodospirillum rubrum*, differs from mammalian cytochrome *c* in that it is not absorbed on Amberlite resin IR-50 in the ammonia form, has a much smaller electrophoretic and ionophoretic mobility at pH 7, possesses an oxidation potential some 80 to 100 mV more oxidizing, and is not activated by mammalian cytochrome *c* oxidase. All of these changes are manifested without any appreciable shifts in the characteristic absorption spectra.

Enzymes associated with cytochrome *c*, such as the diphosphopyridine nucleotide (DPN) linked reductase and cytochrome *c* oxidase, are also found[9,8]. None of the oxidases obtained from these bacteria appear to be identical with those prepared from mammalian tissues. This observation appears to be fairly general for all bacterial oxidases[10].

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** The term "hematin compound" is used as defined by LEMBERG AND LEGGE[1], p. 163.

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A unique "photo-oxidase" for cytochrome *c* has been detected in all the known genera of photosynthetic bacteria and isolated from two of the facultative photoheterotrophs[9,8]. This enzyme exhibits a remarkable heat stability and resistance to cyanide. Together with the reductase and oxidase, it provides a rational basis at the enzyme level for a partial understanding of the known physiological traits of the photosynthetic bacteria, as they range from strict anaerobes to facultative heterotrophs[8].

In addition, a remarkable new type of hematin compound has been observed to occur in all the photosynthetic bacteria available in quantity as fresh mass cultures[7]. Yields obtained on isolation and purification from two facultative heterotrophs indicate that these hematin compounds occur in quantities comparable to those observed for the cytochromes of the "*c*" type. While too little is known about the chemistry and function of these compounds to propose a stable terminology, they have been labeled tentatively "pseudohemoglobins" because of their characteristic spectra, oxidation potential and auto-oxidizability, even though they are not true hemoglobins in that they fail to exhibit oxygen-carrying capacity and react to form hemochromogens characteristic of the cytochromes of "*c*" type[7].

All of these observations dictate the extension of studies, such as the foregoing to as many bacteria as possible, so that the metabolic pattern of hematin compounds in the photosynthetic bacteria can be assessed as influenced by the photochemical act in photosynthesis. In this paper, there are presented the results of researches on hematin compounds in all known genera of the facultative photoheterotrophs (non-sulfur purple bacteria, or *Athiorhodaceae*) as well as in two typical facultative denitrifiers.

METHODS

Cultures and other source materials

(a) *Photosynthetic bacteria*

Rhodospirillum (Rsp) rubrum. This bacterium was represented by the strain, S-1 (Van Niel), used in previous researches[7]. Its culture and manipulation have been described[11]. In addition, it was available in quantities of hundred of grams as a dry powder*, freed of fatty material and photoactive pigments by exhaustive extraction with various organic solvents. This powder, as well as those obtained from other bacteria described below, had been collected by VAN NIEL as part of a project devoted to the study of the bacterial photopigments (chlorophyll, carotenoids, etc.) some 20 years previous to the present researches. Although stored at room temperature the powders (with one exception) were found to be excellent sources of undenatured cytochrome *c* for each of the bacterial species involved. To further attest the remarkable stability of these bacterial cytochromes, it was found they had remained largely in the reduced form during storage through these two decades.

Rhodopseudomonas (Rps) spheroides. In addition to strain 241 (Van Niel) used in previous researches[7], there were also available large quantities of an aged dry powder, prepared from a strain labeled 242 (Van Niel) in the same manner as that described above for *Rsp. rubrum*.

Rhodopseudomonas (Rps) capsulatus (Van Niel). A 10-day culture of this bacterium, grown in 15 liters of a 5% yeast extract-mineral salt medium, was harvested by centrifugation and washed twice with tap water. The yield of fresh cells was 51 g wet weight.

Rhodopseudomonas palustris (Strain 5, Van Niel). 45 g dry powder, as described above for *Rsp. rubrum* were available.

(b) *Chemosynthetic bacteria*

Micrococcus (M) denitrificans. This strain was identical with that studied by KLUYVER AND VERHOEVEN[12]. It could be cultured either autotrophically, using a mineral medium with nitrous oxide, hydrogen and carbon dioxide as substrates, or heterotrophically under either anaerobic or aerobic conditions. The cultures examined for hematin compounds were grown heterotrophically, employing a medium made according to the following prescription: 10 g K_2HPO_4, 20 g KNO_3, 5 ml yeast autolysate, adjusted with conc. H_2SO_4 to pH 6.5–6.8 after making up 1 liter with tap

* See footnote on following page.

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water; after sterilization for 20 min at 120° C, added 12 ml sterile 80% glucose solution. For aerobic cultures, the nitrate was omitted and the medium aerated. It was possible to obtain readily yields of 130 g wet weight fresh cells from 18 liters of a 4–5 day culture.

Pseudomonas (Ps) denitrificans. The strain was that studied by SACKS AND BARKER[13]. It was available as 2.7 g of a dry cell preparation which had been stored frozen (—20° C) for several months. The powder had been prepared from cell suspensions grown anaerobically, with nitrate as H-acceptor.

Biochemical procedures

Extraction methods

The following procedures were used singly, or in combination:

1. Modified KEILIN-HARTREE method, beginning with suspension of cells in warm trichloracetic acid (TCA), as described previously[4]. This was the method of choice for all the isolations of cytochrome *c* components, as well as pseudohemoglobins, from the facultative photoheterotrophs (*i.e.*, *Rsp. rubrum, Rps. spheroides, Rps. capsulatus, Rps. palustris*).

2. Manual grinding in the cold with alumina, or glass beads. The procedure using alumina has been described previously[14]. The alumina powder was #1557 AB (levigated) obtained from Buehler, Ltd., 165 W. Wacker Drive, Chicago, Illinois. The glass beads were available from the Minnesota Mining and Manufacturing Company, St. Paul, Minnesota as "pavement-marking beads", grade 10, sieve size 60/80.

3. Sonic disruption at 0–5° C using a 9 kc Raytheon magnetostriction oscillator.

4. Disruption at 0–5° C in a Waring Blendor using glass beads, as described by LAMANNA AND MALLETTE[15]. This method proved to be the most reliable for general use.

For preparation of enzyme extracts for assay of oxidase and reductase activity, Method No. 4 was invariably employed, except for some facultative photoheterotrophs, which usually were amenable to sonic disruption.

In isolating cytochrome *c* from the bacterial powders, the first step was usually extraction in the cold (0–5° C) with 0.05 *M* phosphate buffer, pH 7.0. This step was repeated once and the combined extracts then fractionated, as in the KEILIN-HARTREE procedure. In all the powders prepared from the facultative photoheterotrophs, little or no hematin component resisted the cold extraction with phosphate. Both the pseudohemoglobin and cytochrome *c* components were usually recovered initially in the ammonium sulfate (AS) fraction between 25 and 50% saturation. On repeating the AS fractionation, the two components gradually separated, exhibiting more usual solubility relations, *i.e.*, the pseudohemoglobin was recovered in a fraction between 25 and 40% saturation, and the cytochrome *c* in a fraction between 50 and 60% saturation. From this point, further purification was achieved by the methods previously described[7]. The final purity was estimated on the basis of relative intensities of α-, β- and γ-bands in the visible and protein absorption in the ultra-violet.

Pyridine and cyanide hemochromogens were prepared as described previously[7].

Spectroscopic measurements

Spectra were determined using a Beckmann DU spectrophotometer. Preparations first were examined before treatment with oxidizing or reducing agents. The spectra of the reduced forms were determined after addition of sodium hydrosulfite. Spectra of oxidized forms were ascertained after addition of potassium ferricyanide, or where feasible, after vigorous aeration. In some cases where isolation was impractical, the presence of hematin compounds was detected by measuring the difference spectra of oxidized and reduced extracts.

Potential measurements

Ferro-ferricyanide oxidation-reduction buffers were used as described by DAVENPORT AND HILL[16]. All pH buffer systems used in these determinations were at a final concentration of 0.02 *M*. The following buffer systems were employed: pH 5, sodium acetate-acetic acid; pH 6, sodium monohydrogen phosphate-potassium dihydrogen phosphate; pH 8 to 9, "tris" (tris-hydroxy-methyl-amino-methane); pH 10–11, sodium tetraborate-sodium hydroxide.

Reagents

DPNH (reduced diphosphopyridine nucleotide) and cytochrome *c* (horse heart) were obtained from the Sigma Chemical Company, St. Louis, Missouri. Cytochrome *c* oxidase from pig heart was made by the Department of Biochemistry, University of California, Berkeley*. All other reagents

* The dried bacterial powders used in these researches were provided us by Professor C. B. VAN NIEL, to whom we are thus doubly indebted. We also wish to record the gift of a dried preparation of *Ps. denitrificans* from Dr. C. C. DELWICHE, Department of Plant Biochemistry, University of California, Berkeley, California. In addition, Dr. M. E. WINFIELD, in residence at the Hopkins Marine Station as a traveling Fellow of the Australian C.S.I.R.O., unselfishly made available to us

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were of C.P. reagent quality, as obtained from commercial sources, and were used without further purification, except for the potassium ferricyanide which was recrystallized twice from water.

Enzyme assays

Only cytochrome *c* oxidase and the DPNH-linked cytochrome *c* reductase activities were assayed in this series of researches. The methods employed have been described previously[8,9].

Preparation of bacterial enzymes

Oxidase and reductase activities in *M. denitrificans* were studied in extracts prepared as follows: 13 g wet weight of fresh washed cells from anaerobic cultures were blendorized with glass beads for 10 minutes (all operations being conducted at 0° C). The cell debris and beads were removed by centrifugation at 1200 *g* for 10 minutes, and discarded. The intact cells remaining in the supernatant liquid were removed by centrifugation at 20,000 *g* for one hour. The supernatant liquid (S) and the residue (H) were both tested for enzyme activity, most of which was found to remain in (S). This "soluble" material was stored at —20° C and used in experiments described below.

Terminology

The system proposed by SCARISBRICK[17] was adopted—thus, the hematin compound was named according to source and location of the α-band of the reduced form. This practice was extended to *Rsp. rubrum* cytochrome *c* as well as other bacterial cytochromes which previously had been referred to as "*c*" cytochromes[7].

RESULTS

Cytochrome c components of the facultative photoheterotrophs

1. *Rsp. rubrum* cytochrome-550. The properties of this compound, as prepared from fresh cell suspensions, have been described[7]. No changes were noted when this cytochrome was prepared from the aged bacterial powders. A yield of approximately 0.2 mg per g dry weight of fresh cells was noted. This yield was approximately 20% of that found using fresh cells.

The spectra characteristic of *Rsp. rubrum* cytochrome-550, as well as those of horse heart cytochrome *c*, are indicated in Table I which exhibits the characteristic spectroscopic data for the various cytochromes of the "*c*" type, obtained from the facultative photoheterotrophs.

2. *Rps. spheroides* (242, Van Niel) cytochrome-550. This cytochrome was obtained in rather poor yields from the powdered material prepared from the strain labeled 242 (Van Niel). Considerably less than 0.01 mg per g dry weight was recovered. However, this material appeared to be identical with the cytochrome *c* obtained in our previous researches using fresh cell suspensions of *Rps. spheroides* (see Table I). It is not known why this low yield occurred in this strain, inasmuch as the yields from the 241 strain were very similar to those noted for *Rsp. rubrum*.

3. *Rps. palustris* cytochrome-552. Yields closely comparable to those found for the *Rsp. rubrum* powder were also noted using powder prepared from *Rps. palustris*. The spectroscopic purity of the material, data for which are presented in Table I, was 25-30%.

4. *Rps. capsulatus* cytochrome-550. Forty g wet weight of fresh cells were employed as starting material. The final yield of cytochrome was approximately 0.5 mg per g dry weight. The purity estimated on the basis of spectroscopic data was 40-50%. The characteristic absorption maxima for this cytochrome, together with those for its cyanide and pyridine hemochromogens, are shown in Table I.

his cultures of *M. denitrificans* and *Rps. capsulatus* and also cooperated in helping us obtain mass cultures of these bacteria. A cytochrome *c* oxidase, prepared from pig heart by the usual KEILIN-HARTREE procedure, was obtained through the courtesy of Professor J. NEILANDS, University of California, Berkeley, California.

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TABLE I

WAVE LENGTHS OF ABSORPTION MAXIMA FOR "*c*" FERROCYTOCHROMES OF PHOTOSYNTHETIC BACTERIA (mμ)

*Compound**	*α-band***	*β-band***	*Soret band***
Rsp. rubrum-cytochrome-550	550	521	416
Rsp. rubrum-cytochrome pyridine hemochromogen	550	520	414
Rsp. rubrum-cytochrome cyanide hemochromogen	554	525	420
Rps. spheroides-cytochrome-550***	550	520	416–417
Rps. spheroides-cytochrome pyridine hemochromogen	550	520	414
Rps. spheroides-cytochrome cyanide hemochromogen	554	525	420
Rps. spheroides-cytochrome-550§	550	519–520	414–416
Rps. capsulatus-cytochrome-550	550	521	416
Rps. capsulatus-cytochrome pyridine hemochromogen	550	520	414
Rps. capsulatus-cytochrome cyanide hemochromogen	555	526	421
Rps. palustris-cytochrome-552	552	523	418
Chromatium D-cytochrome-552	552	525	417–418
Chlorobium limicola-cytochrome-553	553	520	415

* All these cytochromes upon oxidation show the characteristic fusion of the α- and β-bands into a broad band in the green with maximum between 530 and 535 mμ as well as the broadening of the Soret band with shifts toward the blue (maxima at 395–410 mμ).

** Corresponding values (mμ) for mammalian cytochrome *c*; α-band, 550; β-band, 520; Soret band, 416.

*** Isolated from strain 241 (Van Niel).

§ Isolated from strain 242 (Van Niel).

Cytochrome c components of denitrifiers

1. *M. denitrificans*-cytochrome-550. The yield of cytochrome *c* was not appreciably affected by whether the cells were grown under strictly anaerobic, or under aerobic conditions. The cytochrome *c* content appeared to be an order of magnitude less than that encountered in the photoheterotrophs. However, this posed no difficulties, as the amounts of this micro-organism obtainable were so great that quantities of cytochrome sufficient for purification procedures were readily obtained.

It proved necessary to disrupt these organisms by method 4 (see METHODS). Attempts to homogenize the cell suspensions by sonic vibration (with or without glass beads), by manual grinding with various abrasives and by phosphate extraction of acetone powders achieved only 5 to 10% disruption. Blendorizing with glass beads resulted in recovery of 80–90% of the original cell protein in the form of a cell-free extract.

The crude cytochrome resulting from the first AS fractionation was subjected to repeated cycles of AS fractionation until no further purification was obtainable by this means. Further purification by absorption on calcium phosphate gel[7] brought the sample to a spectroscopic purity of 15–20%. This material was then subjected to electrophoretic analysis, using ammonium acetate buffer (0.1 *N*, pH 7.0). Shortly after initiation of electrophoresis, two boundaries appeared both migrating toward the anode. Most of the colored component was concentrated in the slower moving boundary. It was noted that this direction of migration for the cytochrome was opposite to that observed for mammalian cytochrome *c* or *Rsp. rubrum*-cytochrome-550, both of which exhibit

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cathodic mobility at pH 7. Thus, it appeared that *M. denitrificans*-cytochrome-550 was negatively charged at neutral pH, indicating an isoelectric point at acid pH.

The anodic mobility was of an absolute magnitude (10^{-5} cm^2 sec^{-1} $volt^{-1}$) associated with cytochrome *c*.

By continuously displacing the buffer at the anode to compensate for the movement of the faster-moving colorless anodic impurities, it was possible to hold back most of the cytochrome in the cathode compartment. After 5–6 hours, the anode compartment showed little or no color, while the cathode and middle compartments were highly colored—indicating a good separation of the colorless impurities from the cytochrome component. The purest material (in the cathode compartment) exhibited a spectrospopic purity of approximately 50%. This material was used for establishing the nature of the visible absorption spectrum and the oxidation potential. The less pure material from the middle compartment—of spectroscopic purity approximately 35%—was used for preparation of the pyridine and cyanide hemochromogens. Spectroscopic data for wave lengths of absorption maxima are given in Table II.

TABLE II

WAVE LENGTHS (mμ) OF ABSORPTION MAXIMA FOR "*c*" CYTOCHROMES OF DENITRIFIERS

Compound	*α-band*	*β-band*	*Soret band*
(a) Reduced form			
M. denitrificans-cytochrome-550	550	522	416
M. denitrificans-cytochrome pyridine hemochromogen	550	520	414
M. denitrificans-cytochrome cyanide hemochromogen	554	525	420
Ps. denitrificans-cytochrome-552	552	525	418
Ps. denitrificans-cytochrome pyridine hemochromogen	551	520–521	415
Ps. denitrificans-cytochrome cyanide hemochromogen	556	527	422
(b) Oxidized form			
M. denitrificans-cytochrome-550	– (526–528) –		410–411
Ps. denitrificans-cytochrome-552	– (526–528) –		412–413

2. *Ps. denitrificans*-cytochrome-552. 2.7 g of a dry powder were used as starting material. It was found, as with *M. denitrificans*, that blendorizing with glass beads was most effective in extraction of the cytochrome component. However, all of the cytochrome initially precipitated in the first AS fraction (0–25% saturation) rather than at the more usual higher concentrations of AS. Resuspension of the precipitated cytochrome and repeated AS fractionation resulted in normal solubility behavior, *i.e.*, the major fraction of the cytochrome precipitated in the fraction 33–50% saturated AS. This material was refractionated a number of times with AS and finally reached a spectroscopic purity of 10–15%. This preparation was used for establishing the character of the visible absorption spectrum (see Table II for wave lengths of absorption maxima) and for determinations of oxidation potential.

Oxidation potentials of "c" cytochromes

The oxidation potentials at various values of pH for the bacterial cytochromes are shown in Table III. It was noted that the "*c*" cytochromes of the photochemically active bacteria had potentials much more oxidizing than those observed for cytochrome *c*, as

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isolated from yeast or mammalian tissue. (All the values given in Table III were determined in experiments using, as a standard, cytochrome *c* from horse heart, which always gave values for E'_0 in good agreement with the accepted +0.265 v. at pH 7.0.) On the other hand, the nonphotochemical facultative anaerobes (denitrifiers) showed both "normal" and high values. Consideration of the E'_0 values indicated that the cytochrome from *M. denitrificans* might be similar to mammalian cytochrome *c*, whereas the cytochrome from *Ps. denitrificans* appeared to resemble those obtained from the facultative photoheterotrophs.

TABLE III

OXIDATION POTENTIALS (E'_0) OF BACTERIAL CYTOCHROMES OF "*c*" TYPE (volts)

Compound	*pH*					
	5.0	*6.0*	*7.0*	*8.0*	*9.0*	*10.0*
Rps. capsulatus-cytochrome-550	0.33_2	0.33_0	0.32_9	0.32_4	0.31_8	0.31_1
Rps. palustris-cytochrome-552	0.31_0	0.32_7	0.30_7	0.32_0	0.30_4	—
Rsp. rubrum-cytochrome-550	0.38_0	0.35_3	0.33_8	0.30_5	0.30_4	—
M. denitrificans-cytochrome-550*	0.26_5	—	0.25_0	0.25_5	0.22_5	—
M. denitrificans-cytochrome-550**	—	—	0.24_7	—	—	—
Ps. denitrificans-cytochrome-552	—	0.32_0	0.32_0	0.32_0	—	—

* from anaerobic culture.
** from aerobic culture.

It should be noted that *M. denitrificans* cytochrome-550 is the first bacterial cytochrome of "*c*" type to be isolated which shows spectroscopic and electrochemical properties identical with those of mammalian cytochrome *c*. As remarked below, its enzymic properties are also similar to those of mammalian cytochrome *c*.

Enzymic properties of the bacterial cytochromes

Activation by mammalian cytochrome-c-oxidase

The responses of various bacterial cytochromes of the "*c*" type when incubated with a pig heart cytochrome *c* oxidase and air are shown in Fig. 1. It was found that none of

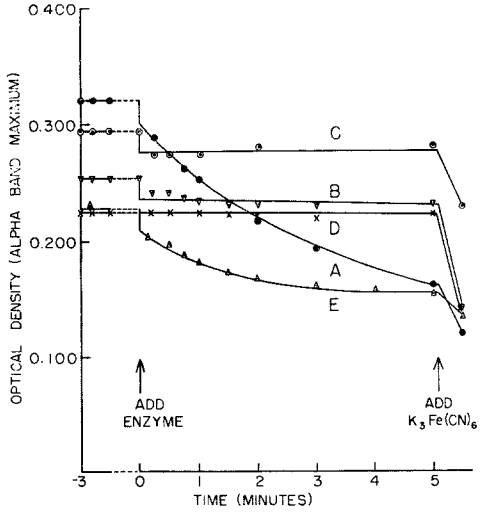

Fig. 1. Effect of pig heart cytochrome *c* oxidase on bacterial cytochromes. Test system: 0.1 *M* phosphate buffer pH 7, 0.1 ml; 0.1% ascorbate-reduced horse heart cytochrome *c*, 0.2 ml, distilled water, 0.3 ml (Curve A). For other curves, water omitted and 0.5 ml each of appropriate cytochromes added as follows: Curve B, *Rsp. rubrum* ferrocytochrome-550; Curve C, *Rps. capsulatus* ferrocytochrome-550; Curve D, *Ps. denitrificans* ferrocytochrome-552; Curve E, *M. denitrificans* ferrocytochrome-550. Total volume of all test systems was 0.8 ml before addition of enzyme (0.2 ml). Experiment commenced, after 3 minute period (arrow). After 5 minutes, potassium ferricyanide added, as indicated. The rate of oxidation was determined by following the change in optical density at the wave length of maximal absorption for the α-band. This wave length was 550 mμ in all cases, except for the *Ps. denitrificans* pigment for which maximal absorption occurred at 552 mμ.

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the cytochromes of high potential tested were oxidized in the presence of the mammalian oxidase. This included not only the samples from photosynthetic bacteria (Curves B and C) but also that from *Ps. denitrificans* (Curve D). The cytochrome from *M. denitrificans*, which exhibited an oxidation potential similar to that of the horse heart cytochrome (Table III), was oxidized readily (Curve E).

Activation by bacterial cytochrome-c oxidases and reductases

It had been shown[8] that the oxidases and reductases derived from either *Rsp. rubrum* or *Rps. spheroides*, could activate cytochrome *c*, whether prepared from mammalian sources or from the bacteria themselves. A similar study of the enzymic activities associated with cytochrome *c*, in extracts of *M. denitrificans*, revealed that the oxidase and reductase could activate both horse heart cytochrome *c* and *M. denitrificans* cytochrome-550. In addition, it was ascertained that *Rsp. rubrum*-cytochrome-550 was oxidized in the presence of the oxidase from *M. denitrificans*.

The relative levels of oxidase and reductase activity in *M. denitrificans* were determined using both horse heart cytochrome *c* and *M. denitrificans*-cytochrome-550. With either cytochrome, closely identical rates were obtained. The oxidase levels were from 3–5 times greater than those for the reductase, after correction for a heat-resistant non-enzymic reducing component which appeared to be present in the bacterial extracts.

"Pseudohemoglobins"

In our previous studies[7], it was noted that both *Rsp. rubrum* and *Rps. spheroides* contained large quantities of a CO-binding heme protein tentatively identified as a "pseudohemoglobin". Evidence for the occurrence of this type of hematin compound was obtained for representative species of other photosynthetic bacteria[7]. In the present studies, "pseudohemoglobin" was recovered in good yield from the *Rsp. rubrum* powder, but none could be detected in the powders of the other facultative heterotrophs available. Examination of fresh cell cultures of *Rps. capsulatus* revealed the presence of a "pseudohemoglobin" apparently closely identical (spectroscopically) with those of the other facultative photoheterotrophs. As in the other "pseudohemoglobins", the "pseudohemoglobin" from *Rps. capsulatus* exhibited an absorption maximum in the reduced form at the same wave length as that for the α-band of the corresponding cytochrome *c* component (Table IV).

TABLE IV

SPECTROSCOPIC PROPERTIES OF "PSEUDOHEMOGLOBINS"

Source	*Absorption maxima (mμ)*	
	Reduced form	*Oxidized form*
Rsp. rubrum	423, 550	393, 490–500, 630
Rsp. rubrum, pyridine hemochromogen	415, 520, 550	406, 530
Rsp. rubrum, cyanide hemochromogen	420, 526, 555	404
Rsp. rubrum, CO-compound	416, 535, 560–565	—
Rps. spheroides (#241)	423, 550	395–400, 490–500, 630
Rps. capsulatus	425, 550	395–400, 495–500, 640
Chromatium	425, 552	400, 490, 630

As will be made apparent (see DISCUSSION), this "pseudohemoglobin" component

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may be of considerable importance for both the light and dark metabolism of the facultative photoheterotrophs, and possibly for the light metabolism of the strict photoanaerobes. It is desirable to adopt a name less likely to cause confusion than "pseudohemoglobin".

DISCUSSION

The results of the present researches re-enforce the previous findings[7,8] that all photosynthetic bacteria contain modified forms of cytochrome *c*, together with a full complement of associated enzymic activities, the levels of which can be correlated with the overall physiology of these bacteria. The presence of other heme proteins, such as cytochromes of the "*b*" type and the new "pseudohemoglobins", indicates that in the facultative photoheterotrophs, at least, a number of electron transport systems are available for coupling with cytochrome *c*[8].

Spectroscopic examinations by DUYSENS[19] of actively metabolizing cell suspensions of *Rsp. rubrum* have indicated that the major effect of illumination under normal physiological conditions is photo-oxidation of a cytochrome component with a difference spectrum (oxidized-reduced) very similar to that expected to arise owing to oxidation of a modified form of cytochrome *c*. More detailed observations by CHANCE AND SMITH[20] working with cell suspensions of *Rsp. rubrum*, incubated under a variety of conditions (anaerobic, aerobic, presence of respiratory inhibitors), have shown that the action spectrum is complex, appearing to arise from photo-oxidation of the whole cytochrome system, including a major contribution from a CO-binding pigment which has spectroscopic properties identical with those of the *Rsp. rubrum* "pseudohemoglobin"-550. It will be recalled that in the previous studies[8] it has been postulated that the shift from light to dark metabolism in the facultative photoheterotrophs involves a competitive interaction between two oxidase systems. One of these is light activated (photo-oxidase) and cyanide-insensitive. The other is light-insensitive and cyanide-sensitive. The steady state oxidation level of the bacterial cytochrome *c* is correlated with the relative levels of these oxidase activities in the various photoheterotrophs. CHANCE AND SMITH[20] suggest a similar competition mechanism. However, they propose the CO-binding pigment as the terminal oxidase in the dark metabolism and as the focal point for light mediation of the electron transport by cytochromes. Inasmuch as it has been shown that these heme proteins are capable of coupling enzymically or nonenzymically in a number of ways[8] it is not possible to deduce an unambiguous pathway for electron transport from the data available at present. In particular, the locus for introduction of the excitation energy absorbed through chlorophyll remains uncertain.

In connection with extension of this "cytochrome-mediation" hypothesis to photosynthesis in general, it is pertinent to examine data on (a) the distribution of hematin compounds in all photosynthetic systems, and (b) occurrence of enzymic activities associated with hematin compounds in these systems. Comparisons should also be made, where possible, with non-photosynthetic systems.

As regards (a), it may be stated with reasonable certainty that all photosynthetic systems examined to date contain a cytochrome *c*, which differs physically and chemically, and is distinguishable enzymically from the typical cytochrome *c* encountered in mammalian tissue or in yeast. It is less certain whether cytochromes of the "*b*" type are invariably associated with photosynthetic systems. The presence of the "pseudo-

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hemoglobins" has been established in all the photosynthetic bacteria available in fresh mass cultures. It is not known whether corresponding hematin compounds are present in green plant tissues. However, the occurrence of cytochrome "*f*", which may be considered a modified cytochrome *c*, appears to be quite general in green plants[16]. Thus, no data are in existence which contra-indicate the suggestion that a system of hematin compounds, modified from those encountered in mammalian tissues or in yeast, is a characteristic component of photosynthetic tissues.

Data on point (b) indicate that reductases and photo-oxidases for bacterial "*c*" cytochromes are found in all the photosynthetic bacteria, regardless of their ability to tolerate or utilize oxygen[8]. It is not clear what corresponds to a photo-oxidase in the green plant. However, data have been presented by LUNDEGÅRDH[21] which are claimed to provide evidence for photo-oxidation of cytochrome *f* during active photosynthesis in green plants and algae. The outstanding difference between bacterial and green plant photosynthesis is the production of molecular oxygen in the latter. This may be rationalized by supposing that the "photo-oxidase" in the green plant chloroplast is itself a catalase or is coupled with a catalase, whereas the bacterial photo-oxidase is coupled with a peroxidase.

In considering comparable phenomena in non-photosynthetic systems, it should be remarked that the photosynthetic bacteria are potentially facultative anaerobes in the sense that the photochemical "OH" is an oxygen substitute in electron transport, much as is sulfate or nitrate in the metabolism of non-photosynthetic facultative anaerobes. It may be noted that in the present researches cytochromes of "*c*" type have been isolated from two typical denitrifiers. Also, the existence of a cytochrome—apparently of the "*c*" type—in the strictly anaerobic sulfate reducer, *Desulfovibrio desulfuricans**, has been demonstrated by POSTGATE[22]. In these three instances, two-*Ps. denitrificans* and *Desulfovibrio*—yield cytochromes with spectra modified from those typical of the mammalian form. One—*M. denitrificans*—contains a cytochrome *c* spectroscopically identical with the mammalian compound.

All the cytochromes of "*c*" type from the photosynthetic bacteria, the oxidation potentials of which have been examined, appear to be "high-potential" cytochromes (Table III). This finding lends added significance to the observation by DAVENPORT AND HILL[16] that the chloroplast cytochrome (cytochrome *f*) also is a high potential cytochrome. It will be necessary to extend the present researches to the cytochromes of the photo-anaerobes such as *Chromatium* and *Chlorobium*—to test the generality of occurrence of high potential cytochromes in photosynthetic systems. A similar extension to non-photosynthetic bacteria—particularly the interesting cases of facultative anaerobes—has been effected in only two micro-organisms, the denitrifiers studied in the present researches. It is found that *Ps. denitrificans*- cytochrome-552 is a high potential cytochrome, whereas *M. denitrificans*-cytochrome-550 is not.

These observations can be correlated with the physiology of these two bacteria. *Ps. denitrificans*, when grown in the absence of oxygen in nitrate-glucose medium, contains much more of its cytochrome *c* than when grown aerobically[23]. It has been noted by SACKS AND BARKER[13] that cell-free extracts show characteristic absorption peaks at 552 and 522 mμ, in agreement with the values for the α- and β-bands of the reduced

* One of us (L.P.V.), has isolated an auto-oxidizable cytochrome, apparently of the "*c*" type, from a strain of *Desulfovobrio desulfuricans*. The absorption maxima of this hematin compound in the reduced form are at the same wave lengths as reported by POSTGATE[22].

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Ps. denitrificans-cytochrome-552 found in the present researches. In intact cell suspensions, it has been shown[23] that these peaks disappear on aeration and appear when oyxgen is removed. Further, addition of KNO_3 or KNO_2 (in the absence of oxygen) causes these bands to fade; after complete utilization of the KNO_2 the bands re-appear. Finally, KCN is effective in preventing this oxidation of cytochrome by KNO_2, while still permitting reduction by the extract (presumably through a DPN-linked reductase of the type found in the present researches in *M. denitrificans*.)

These observations provide a rational basis, if not conclusive proof, for supposing that *Ps. denitrificans*-cytochrome-552 mediates reduction of nitrate and nitrite, in a manner analogous to that postulated for the reduction of "OH" by *Rsp. rubrum*-cytochrome-550 in photo-metabolism[8].

On the other hand, it is observed in the present studies that no change in character or amount of cytochrome *c* occurs in *M. denitrificans*, whether grown aerobically or anaerobically. It appears that there is no adaptive response to nitrate, or the necessity to reduce nitrate, in *M. denitrificans* as there is in *Ps. denitrificans*. This raises the possibility that reduction of nitrate does not go through cytochrome *c* in *M. denitrificans*, whereas it appears to be a favored or required pathway in *Ps. denitrificans*.

Evidence has been presented by POSTGATE recently[24] which indicates participation of the cytochrome-*c*-like compound in sulfate reduction by *Desulfovibrio*. If this is true, it would appear that the oxidation potential of this cytochrome must be considerably less positive than most cytochromes of the "*c*" type, because any reasonable guess as to the oxidation potential of the sulfate-sulfite couple, or for reduction of sulfate to other lower valence forms of sulfur, indicates a value some 100 to 150 mV less oxidizing than the normal value for mammalian cytochrome *c*[25].

It is evident that more insight into the factors which govern the oxidation potentials in the various hematin compounds is required before a coherent picture of electron transport involving such compounds can be proposed. The availability of a much greater variety of cytochromes of the "*c*" type as well as of the new hematin compounds found in the present researches should facilitate further study.

It is particularly important to investigate the spectra in the near infra-red of the bacterial hematin compounds, and also of cytochrome *f*, because the nature of these spectra bears directly on the question of whether excitationen ergy from chlorophyll can be transferred directly to the hematin compounds. Thus, the existence of absorption bands in the bacterial cytochromes and other hematin compounds which lie just beyond the farthest emission frequencies of the bacterial chlorophylls (> 900 mμ) would indicate strongly that direct transfer by a process such as inductive resonance[26] could occur. In a similar fashion, the existence of absorption bands in cytochrome *f* in the region beyond 690 mμ would be of significance.

If it is demonstrated that light energy affects metabolism primarily through the cytochrome system, then it will be necessary to elaborate mechanisms to explain the eventual appearance of electrons at the potential of the hydrogen electrode. A scheme for achieving this by coupling of oxidized and reduced cytochrome systems has been suggested by DAVENPORT AND HILL[16]. It is evident there should be increasing attention to the necessity for adapting what is known about hematin chemistry to the problem of energy transfer in photosynthesis, even if such speculations run far ahead of the data available.

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SUMMARY

1. All photosynthetic bacteria appear to contain cytochromes of the "*c*" type together with associated enzymic activities, such as DPNH-linked reductase, oxidase and photo-oxidase. This is true of strict anaerobes as well as facultative bacteria. There also appears to be a general distribution of other hematin compounds, such as cytochromes of the "*b*" type, as well as of the new hematin compounds tentatively referred to as "pseudohemoglobins".

2. Two species of denitrifiers (facultative anaerobes) have also been examined and found to contain cytochromes of the "*c*" type. One of these species yields a cytochrome *c* which spectroscopically and enzymically resembles mammalian cytochrome *c*, whereas the other contains a cytochrome very similar in spectroscopic properties, oxidation potential and enzymic behavior to the cytochromes of "*c*" type found in photosynthetic bacteria.

3. The oxidation potentials of the various cytochromes of "*c*" type have been measured over a wide range of pH values. These results indicate that each microorganism examined has a cytochrome *c* unique to itself, but that all the "*c*" cytochromes isolated from the facultative photoheterotrophs have potentials much more oxidizing than those of cytochrome *c* as prepared from yeast or mammalian tissue.

4. These results are discussed and correlated with the overall physiology of the bacteria. Their significance for the general problem of transfer and storage of light energy in photosynthesis is indicated.

RÉSUMÉ

1. Toutes les bactéries photosynthétiques renferment des cytochromes de type "*c*" associés à des activités enzymatiques, telles que réductase liée au DPNH, oxydase et photo-oxydase. Ceci est vrai pour les anaérobies stricts aussi bien que pour les anaérobies facultatives. Il apparaît qu'il y a aussi une distribution générale d'autres composés hématiniques, tels que des cytochromes du type "*b*", aussi bien que des nouveaux composés hématiniques qu'on pourrait désigner par le terme de "pseudohémoglobines".

2. Deux espèces de bactéries dénitrifiantes (anaérobies facultatives) ont également été étudiées et se sont révélées contenir des cytochromes du type "*c*". L'une de ces espèces produit un cytochrome *c* qui ressemble du point de vue spectroscopique et du point de vue enzymatique au cytochrome *c* des mammifères, tandis que l'autre contient un cytochrome dont les propriétés spectroscopiques, le potentiel d'oxydo-réduction et le comportement enzymatique sont très voisins de ceux des cytochromes du type "*c*" trouvés chez les bactéries photosynthétiques.

3. Les potentiels d'oxydo-réduction des divers cytochromes du type "*c*" ont été déterminés dans un domaine étendu de pH. Les résultats montrent que chaque micro-organisme examiné possède un cytochrome *c* qui lui est propre, mais que tous les cytochromes "*c*" isolés de photohétérotrophes facultatifs ont des potentiels beaucoup plus oxydants que ceux des cytochromes *c* qu'on peut préparer à partir de la levure ou des tissus de mammifères.

4. Les résultats sont discutés en relation avec la physiologie globale des bactéries. Leur signification pour le problème général du transfert et du stockage de l'énergie lumineuse au cours de la photosynthèse est indiquée.

ZUSAMMENFASSUNG

1. Alle photosynthetischen Bakterien scheinen Cytochrome vom "*c*"-Typus, sowie damit verbundene Enzym-Aktivitäten, wie z.B. $DPNH_2$-Reduktase, Oxydase und Photooxydase zu enthalten. Das gilt sowohl für strenge Anaerobier, als auch für bedingt anaerobe Bakterien. Andererseits scheinen auch andere Hämatinverbindungen, wie Cytochrome des "*b*"-Typus, sowie die neuen Hämatinverbindungen, die versuchsweise als "Pseudohämoglobine" gekennzeichnet wurden, allgemein vorzukommen.

2. Zwei denitrifizierende Arten (bedingt anaerob) wurden ebenfalls geprüft und festgestellt, dass auch sie Cytochrome vom "*c*"-Typus enthalten. Eine dieser Bakterien liefert ein Cytochrom *c*, welches spektroskopisch und enzymatisch dem Cytochrom *c* aus Säugetiergeweben ähnelt; andere enthalten ein Cytochrom, welches in seinen spektroskopischen Eigenschaften, seinem Oxydationspotential und seiner Enzymwirkung denen ähnelt, welche in photosynthetischen Bakterien gefunden wurden.

3. Die Oxydationspotentiale verschiedener Cytochrome vom "*c*"-Typus wurden über einen weiten pH-Bereich gemessen. Die Ergebnisse lassen vermuten, dass jeder untersuchte Mikroorganismus ein artspezifisches Cytochrom *c* besitzt, dass aber andererseits die "*c*"-Cytochrome, die aus bedingten Photoheterotrophen isoliert wurden, ein viel höheres Oxydationspotential besitzen, als die aus Hefe oder aus Säugetiergeweben gewonnenen Cytochrome.

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4. Diese Ergebnisse werden diskutiert und mit der allgemeinen Physiologie der Bakterien in Beziehung gebracht. Ihre Bedeutung für das allgemeine Problem der Übertragung und Speicherung der Lichtenergie in der Photosynthese wird erwähnt.

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